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REVIEW ARTICLE

In-Vitro Methods for Evaluation of Antimicrobial Activity

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ABSTRACT

Medicinal plans having different therapeutic activities such as anti-inflammatory, antimicrobial and anti-oxidant. These activities are evaluated by different methods by using various solvents. The present review focused on in vitro methods for evaluation of anti-microbial activity. Various methods are used to screening of antimicrobial activity in medicinal plants. Most commonly used in vitro methods are agar disk diffusion method, Bio autography or thin layer chromatography and broth dilution methods were used. By using these methods rapidly identify the antimicrobial activity in related products.

Key words: Anti-microbial activity, In vitro, agar disk diffusion, Broth dilution, Bio autography

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1. Introduction

The inhibition of microbial growth under standardization conditions may be utilized for demonstrating the therapeutic efficacy of antibiotics. Any change in the antibiotic molecule which may not be detected by chemical methods will be revealed by the anti-microbial activity and hence the microbiological assays are very use full for resolving doubts regarding possible loss of potency of antibiotics[1].

Antimicrobial activity in plants

With the advancement in Science and Technology, remarkable progress has been made in the field of medicine with the discoveries of many natural and synthetic drugs. Antibiotics are undeniably one of the most important therapeutic discoveries of the 20th century that had effectiveness against serious bacterial infections. However, only one third of the infectious diseases known have been treated from these synthetic products6. This is because of the emergence of resistant pathogens that is beyond doubt

the consequence of years of widespread indiscriminate use, incessant and misuse of antibiotics. Antibiotic resistance has increased substantially in the recent years and is posing an ever increasing therapeutic problem. One of the methods to reduce the resistance to antibiotics is by using antibiotic resistance inhibitors from plants. Plants are known to produce a variety of compounds to protect themselves against a variety of pathogens. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant pathogens. Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world. Hence, researchers have recently paid attention to safer phytomedicines and biologically active compounds isolated from plant species used in herbal medicines with acceptable[2,3].

Common terms used in antimicrobials

Bactericidal:

It is defined as a chemical agent capable of killing bacteria, but not necessarily bacterial spores.

Bacteriostatic:

It is defined as the chemical agent capable of preventing the growth of bacteria but not of killing them. Here reproduction and replication is prevented.

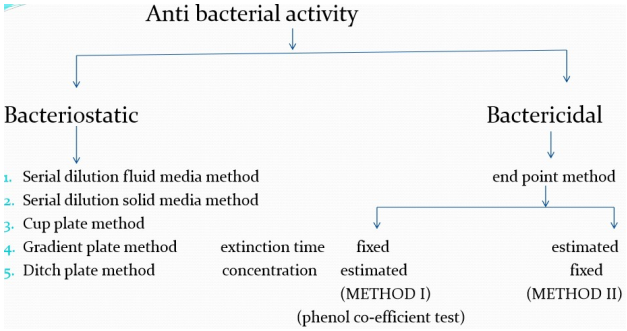
Minimum Inhibitory Concentration (MIC):

It is defined as the lowest concentration of the drug or antibiotic that inhibits the growth of the test organism.

Minimum Bactericidal Concentration (MBC):

It is defined as the minimum concentration of the drug or antibiotic that kills the given test organism.

In vitro methods for Screening of Antibacterial activity:



2. Assessment of Bacteriostatic Activity

- Serial dilution in fluid media
- Serial dilution in solid media
- Cup plate methods
- The gradient-plate method
- The ditch-plate technique

Serial dilution in fluid media:

In this method, graded concentrations of the test substance in a nutrient medium are inoculated with the test organism and incubated. The minimum concentration preventing detectable growth (MIC) is taken as a measure of bacteriostatic activity[4].

Serial dilution in solid media:

A suitable volume of double strength nutrient agar is diluted with an equal volume of bacteriostatic solution and poured into a sterile petridish. When solidified the surface is dried by incubating at 37^0C. Drops of 24hrs broth culture of the test organisms are placed on the dried surface and incubated for 2 to 3 days. Upto 27 cultures can be tested on each plate if a multi-point inoculator is used. This method is mainly used for solutions which give turbidity with fluid nutrient media[4,5].

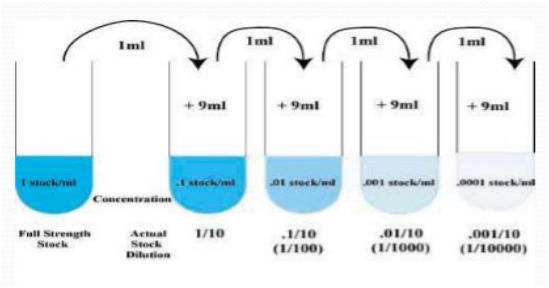


Fig 1: Serial dilution fluid media

Cup-plate method:

In these methods the agar is melted, cooled suitably, inoculated with the test organism and poured into a sterile petri dish. In the cup-plate method, when the inoculated agar is solidified, holes about 8mm in diameter are cut in the medium with a sterile cork borer. In all cases zones of inhibition may be observed, the diameter of the zones giving a rough indication of the relative activities of different anti-microbial substance.

The gradient-plate method:

Two layers of agar is poured. The plates are then incubated over night to allow diffusion of anti-microbial substance. The agar is streaked in the same line as the slope of the agar and reincubated. An approximate MIC can be obtained from the following equation:

MIC = C (x/y) mg/ml

Where, C = concentration in mg/ml, in total volume

X = length of growth, in cm,

Y = total length of possible growth, in cm

The ditch-plate technique:

An agar is poured in a petriplate, allow to solidify, and ditch cut out is made of the agar. A solution of the antimicrobial substance or a mixture of this with agar is carefully run into the ditch so as to about three-quarters fill it. A loopful of each test organism is then streaked outwards from the ditch on the agar surface. Organisms resistant to the antimicrobial grow right up to the ditch whereas susceptible organisms show a zone of inhibition adjacent to the ditch. The width of the inhibition zone gives an indication of the relative activity of the antimicrobial substance against the various test organisms.

Assessment of bactericidal activity

There are two types of extinction time method;

Method 1: A Phenol coefficient type test in which the extinction time is fixed and the concentration of disinfectant needed to kill in the specified time is estimated[6].

Method 2: In which the concentration of bactericide is fixed and the extinction time is estimated.

Phenol Co-Efficient Test:

- Rideal walker test
- Chick martin test

Rideal walker test:

Principle:

Dilutions of the test disinfectant is compared with the standard dilutions of the phenol (usually 1 in 95 to 1 in 115) further activity against salmonella typhi.

Procedure:

Take 24 hrs culture of the S. typhi. Test disinfectant solution or phenol is added about 0.2 ml to the S. typhi culture of 24 hrs. Atintervals of 2.5, 5, 7.5, 10 min sub cultures are taken and transferred to the fresh broth media. The broth tubes are incubated at 37^0c for 48-72 hours and growth is measured.

$$\text{Rideal walker} = \frac{\text{dilution of test disinfectant killing in 7.5min not in 5 min}}{\text{co-efficient}}$$

Dilution of the standard phenol killing in7.5 min not in 5 min

Chick martin test:

Principle:

This test is carried in the presence of organic matter like 3% human faecas or dried yeast.

Procedure:

Serial dilutions of test solution and phenol is prepared in distilled water and 3% yeast suspension is also added. S typhi organsim is added to the above solution. After contact time of 30 minutes the above mixture is transferred to the freshly prepared 10 ml of broth and the test tubes are incubated at the 370c for 48 hours. After the incubation Presence or absence of the growth is calculated.

$$\text{Chick martin} = \frac{\text{Mean of highest phenol conc inhibiting and lowest growth Co-efficient}}{\text{Mean of highest disinfectant conc inhibiting and lowest permitting growth}}$$

Other method for Screening of Antimicrobial activity

Most commonly used in vitro methods for evaluation of antimicrobial activity such as diffusion method, broth dilution and Bio autography.

Diffusion methods:

Agar well diffusion method:

Agar well-diffusion method was followed to determine the antimicrobial activity. Nutrient agar (NA) and Potato Dextrose Agar (PDA) plates were swabbed (sterile cotton swabs) with 8 hour old - broth culture of respective bacteria and fungi. Wells (10mm diameter and about 2 cm a part) were made in each of these plates using sterile cork borer. Stock solution of each plant extract was prepared at a concentration of 1 mg/ml in different plant extacts viz. Methanol, Ethanol, Petroleum Ether, Water. About 100 µl of different concentrations of plant solvent extracts were added sterile syringe into the wells and allowed to diffuse at room temperature for 2hrs. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37°C for 18-24 h for bacterial pathogens and 28°C for 48 hours fungal pathogens. The diameter of the inhibition zone (mm) was measured and the activity index was also calculated. Triplicates were maintained and the experiment was repeated thrice, for each replicates reading were taken in three different fixed directions and average values were recorded[8,9].

Paper disc diffusion assay

The dried plant extract was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 100 mg/mL. DMSO was used as a negative control and 1 mg/mL of Chloramphenicol was used as a positive control for this assay. Sterile filter paper discs 5 mm in diameter, were impregnated with 2 mg (15 µL) of plant extract. Escherichia coli and Bacillus subtilis were used to determine the antibacterial activities. Cultures of E.coli and B. subtilis were stored in CASO-Bouillon broth with 70% glycerol (Acros organics, Geel, Belgium) at -80 °C before inoculating 50 mL of CASO-Bouillon broth, and incubating 37 °C overnight. 250µL of bacterial cell suspension (at a concentration of 108 CFU/mL) was spread onto the surface ofCASO-Bouillon agar media in 9 cm diameter Petri dishes before additional of the impregnated papers discs. The diameter of an inhibition zone around the discs was measured after incubating bacterial plates at 37 °C in the dark for 24 h. The values were recorded with the average (mm) of two diameter measurements per disc taken in two directions, roughly perpendicular. This assay was done in 5 replicates.

Agar well diffusion assay:

The principle of agar well diffusion is similar to that of agar disk diffusion assay. A standardized concentration of inoculum with fixed volume is spread evenly on the surface of gelled agar plate. A hole which ranges from 6 - 8 mm in diameter is punched with a sterile cork borer aseptically in the middle. A fixed volume of plant extractis then introduced into the bored agar well and incubated at optimum temperature and duration depending upon the test microorganism[10].

Poison food technique:

Generally antifungal activity is determined by poisoned food technique. Five-day old fungal culture is punched aseptically with a sterile cork borer of generally 7mm diameter. The fungal discs are then put on the gelled agar plate. The agar plates have been prepared by impregnating desired concentration of plant extract at a temperature of 45 - 50°C. The plates are then incubated at temperature 26 ± 1°C for fungi. Colony diameter is recorded by measuring the two opposite circumference of the colony growth. Percentage inhibition of mycellial growth is evaluated by comparing the colony diameter of poisoned plate (with plant extract) and nonpoisoned plate (with distilled water) and calculated using the formula given below,

$$\%\ \text{of Mycellial inhibition} = \frac{\text{Mycellial groth (Control)} - \text{Mycellial (treatment)}}{\text{Mycellial groth (Control)}} \times 100$$

Spore germination assay

In addition to the above mentioned assays, antifungal activity of plant extracts can be evaluated by spore germination assay using the slide technique. Plant extract of desired concentration and volume are added to the surface of dried slides as a film or in a cavity of a cavity slide. Fixed volume and standard concentration of spore suspension of test fungi are spread over the film whereas in controlled treatment, distilled water is added in place of spore suspension. Slides are then placed on a glass rod in Petri dish under moistened conditions and incubated for

24°C. After incubation, slides are fixed in lacto phenol cotton blue and observed microscopically for spore germination. Percentage spore germination is calculated according to the following formula.

$$\% \text{ Spore Germination} = \frac{\text{Germinated spores (No.)}}{\text{Total Spores (No.)}} \times 100$$

Broth dilution methods

Broth micro dilution assay:

Microorganism spores were grown on a CM agar plate for 3 days at 30 °C until sporulation. A spore suspension was created by adding physiological salt (0.9% NaCl) to the plates and lightly scraping the surface of fungal growth. The spores were collected and pipetted to mira cloth for removing the fungal mycelium. The spore suspension was then diluted and the number of spores counted per mL using hemocytometer. Stock of microorganism spores was made at 107 CFU/mL and kept refrigerated at 4 °C. Spore stock can be used for two weeks after harvesting.

The microplate assay was done in a 96 well microplate. 200 µl of plant extract (200 µg/mL) was added to the first well and twofold dilutions were made with sterile water to concentrations of 100, 50, 25 and 12.5 µg/mL. A 100 µL hydrogen peroxide (H2O2) solution (80 mM) was used as the positive control to make a concentration of 40 mM, while 100 µL sterile water and DMSO (concentrations of 2.5, 1.25, 0.625 and 0.312 %) were used as the negative controls. The stock spores were diluted in CM to a concentration of 2 x 105 CFU/mL before adding into each well. The wells were inoculated with 100 µL of spore stock to have the final concentration of 105 CFU/mL. The total volume in each well is 200µL. The microplate was incubated at 37 °C in the dark, and measured every hour for 40 hours by a microplate reader. Each well absorbance was measured invidually by using spectrophotometry. This assay was done in 4 replications[10,11].

Broth macro dilution assay:

The basic principle of this assay is the same as the broth microdilution assay. But the test is performed in a test tube. In macrodilution assay, a set of test tubes with different concentrations of plant extract with the same volume are prepared. Tubes are inoculated with test microorganisms of standard concentrations as discussed above. After incubation, tubes are examined for changes in turbidity as an indicator of growth. MIC of the plant extract or the test phytochemical can be determined using the above discussed methods.

3. Bioautography

Bioautography is a very convenient way of testing plantextracts and pure phytochemical compounds for their effect on both human pathogenic and plant pathogenic microorganisms. It can be employed in the target directed isolation of active constituents. Bioautography is also employed as a preliminary phytochemical screening technique, by bioassay guided fractionation, to detect active components[12,13]. Paper chromatography followed by bioautography was used for the first time in 1946 by Goodal and Levi to estimate the purity of penicillin. In this method, developed paper chromatogram was placed onto the inoculated agar layer enabling the diffusion of antibiotics from paper to agar containing microorganisms. Thin layer chromatography - bioautography was introduced by Fisher and Lautner et al., 1961. Bioautography methods are usually grouped into three categories, agar diffusion or contact bioautography, immersion or agaroverlay bioautography and direct bioautography (Rios et al., 1988).

Bioautographic method direct-variant (chromatogram layer): Direct variant of the bioautographic method carried out in this work is outlined as follows:

- preparation and application of natural products on thin layer chromatography plates (TLC)
- preparation and application of the bacterial inoculum to TLC plates
- Incubation; and
- Growth detection by colorimetric assay (INT) and measurement of growth inhibition diameters.

In the first step, 10 mL of extracts, fractions and pure substances (dissolved and diluted as mentioned previously) were applied to TLC plates as a spot corresponding to 400, 200 and 100 mg, respectively. The natural products which displayed some activity were diluted to concentrations that varied from 400 to 50 mg for extracts, 200 to25 mg for fractions and 100 to 12.5 mg for pure substances. Each sample spot was located about 2 cm apart and away from the bottom of TLC plate. Sample spots were performed with a micropipette, thus the spot diameter was about 4 mm. One 6 X 6 cm TLC plate was used for each test with four test-samples; 20 mg of chloramphenicol dissolved in 10 mL of DMSO and 10mL aliquots of solvents were applied to plates as control. In step 2, bacterial inoculum was prepared as previously mentioned and transferred to a sterile Petri plate.

The TLC plates loaded with the natural products were covered twice with bacterial suspension for 5 s. Excess of suspension was removed and the TLC plate placed into another sterile Petri plate. In step 3, systems were incubated for 24 h at 36ºC ± 1ºC inside a hermetically closed polyethylene box. A Becker flask containing a water embedded cotton ball was placed beside the plates for keeping the air inside under moist conditions. In step 4, TLC plates were sprayed with 1 mL salt solution of p-iodonitrotetrazolium violet (INT). Plates were incubated for more 4 h and the inhibition diameter zones were observed and measured in mm hour after hour. The development of bioautographic method direct variant, we performed tests with 24 and 48 h grown culture of Staphylococcus aureus and with three different indicator solution concentrations (INT) to establish appropriate conditions for the execution of this method. Tests using bacterial inoculum and INT concentrations were performed in triplicate[14].

Bio autographic method indirect-variant (agar diffusion): In this procedure, first step corresponded to bio autographic variant-direct step 1. In step 2, TLC plates were covered with Mueller-Hinton agar layer (9 mL of the medium on 81 cm2 petri plate area). However, contact between bacterial suspension and natural products were performed by two distinct procedures: mixing with agar

(100 mL test-bacterial suspensions were mixed with 9 mL of agar and carefully poured on TLC plate) and swabbing with a cotton swab (inoculum was spread on the agar surface as described previously). Only the extracts evaluated in this procedure were tested with two types of bacterial inoculum and all tests were carried out in duplicate. In order to compare between the two variants of bio autographic method, results obtained with the use of "pour plate" technique (bacterial suspension mixed with agar) were validated[15].

Immersion or agar overlay bio autography:

In this method the chromatogram is covered with a molten, seeded agar medium. After solidification, incubation and staining (usually with tetrazolium dye), the inhibition or growth bands are visualized. Agar overlay is a hybrid of contact and direct bioautography.

4. Conclusion

Thousands of plant constituents it exhibit the medicinal properties like anti-microbial anti-inflammatory and anti-oxidant properties. Screening of medicinal property in natural products by using various in vitro methods. The present review describes that in vitro methods for anti-microbial activity by using various techniques such that agar diffusion methods, broth dilution method and bio autography. To evaluation of anti-microbial activity used to the suitable microorganism and nutrient broth. Advantage of this method to standardize methods of extraction and in vitro antimicrobial efficacy testing helpful for the invention of new biologically active compounds.

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